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## BACKGROUND OF INVENTION

E-selectin and P-selectin are expressed on activated endothelial cells (EC's). P-selectin also is expressed on activated platelets. Both selectins play roles in various phases of cell interactions, such as, the inflammatory response.

P-selectin is localized at (i) Weibel-Pallade bodies present in the cytoplasm of resting EC's and (ii) α-granules of resting platelets. When EC's or platelets are activated by various factors (e.g. thrombin, ADP, phorbol esters, histamine and free radical oxygen [$O_2^-$]), Weibel-Pallade bodies or α-granules are translocated rapidly to the EC or platelet surface, leading to P-selectin expression. The exact mechanism of such translocation is not well understood, but likely involves a number of transmembrane signaling mechanisms, e.g. those mediated by protein kinase C, thromboxane and eicosenoids. The translocation/expression process is rapid (takes only 1-3 minutes).

In contrast, expression of E-selectin at the EC surface, which results, for example, from stimulation by TNFα and IL-1ß, requires de novo synthesis of E-selectin, i.e. a 4-5 hour "lag time" between stimulation and expression.

P-selectin is believed to be involved in the initial rapid adhesion of neutrophils to EC's, while E-selectin is believed to be involved in subsequent reinforcement of that adhesion. Both processes are important in mediation of the inflammatory response.

neutrophils to EC's is considered to be an important step in the process of neutrophil recruitment and accumulation at inflammatory sites resulting from wounding, infection, or blocking of blood circulation (thrombosis). The major damage from the inflammatory response results from accumulation of neutrophils which produce $O_2^-$ and $H_2O_2$, which in turn cause serious tissue damage. For example, the major tissue damage following heart attack or brain hemorrhage (stroke) results from neutrophil migration and accumulation in tissues, rather than from ischemia (blocking of blood supply). An example is the "reperfusion injury" which occurs when a thrombosis is eliminated by specific treatment and blood circulation is restored. As a consequence of reperfusion, many neutrophils migrate out of the capillaries into surrounding tissues, damaging tissue structure and function.

Immediately after the overall sequence of selectins was clarified through cDNA cloning, and the presence of a C-type lectin domain at the N-terminal domain of both P-selectin and E-selectin was demonstrated (for example, 1 and 2), many undertook an intensive search for the carbohydrate epitopes recognized by those selectins.

$SLe^x$ has been considered to be a plausible ligand of P-selectin and E-selectin based on the following observations: (i) transfection of Lewis fucosyltransferase cDNA in Chinese hamster ovary (CHO) cells expressing sialosyl type 2 chain resulted in acquisition of the ability to adhere to TNFα-activated endothelial cells (3); (ii) HL60 cells, previously shown to react with mAb FH6, are capable of binding to TNFα-activated or IL-1-activated EC's, and the binding can be inhibited by liposomes containing $SLe^x$-bearing GSL's

sialosylparagloboside, sialosylnorhexaosylceramide or $Le^x$-glycosylceramides; (iii) mAb's SNH3 and SNH4 inhibited E-selectin-dependent HL60 cell adhesion (4); and (iv) subsequent confirming studies utilized other anti-$SLe^x$ mAb's, oligosaccharides or GSL's containing the $SLe^x$ structure.

Some studies indicated that selectin-dependent binding, particularly in tumor cells, also is mediated by $SLe^a$ (a positional isomer of $SLe^x$) (5-7). However, $SLe^a$, which has a lacto-series type 1 chain structure, is completely absent from human neutrophils and HL60 cells.

Based on antibody reactivity, $SLe^x$ is thought to be expressed in the form of O-linked, N-linked or lipid-linked carbohydrate chains.

Although many selectin-related studies since have been published, those studies all were based on inhibition by or adherence to only a suspected structure. There has been almost no effort directed to elucidating the chemical isolation and characterization of the real carbohydrate target structure of selectins present in normal human neutrophils or HL60 cells, because of the extreme difficulty of isolating and characterizing the essential epitope expressed in those cells.

Tiemeyer et al. (8) isolated the VIM-2 antigen structure from a relatively large quantity of HL60 cells. VIM-2 has the structure,

$$\begin{array}{l}\mathrm{NeuAc\alpha2{\rightarrow}3Gal\beta1{\rightarrow}4GlcNAc\beta1{\rightarrow}3Gal\beta1{\rightarrow}4GlcNAc\beta1{\rightarrow}} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad\ \ 3 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad\ \ \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad\ \mathrm{Fuc\alpha1}\end{array}$$

and was believed to be the E-selectin binding site. However, Lowe et al. (9) failed to observe E-selectin-dependent adhesion of VIM-2-positive, $SLe^x$-negative CHO cells and therefore were unable to confirm the role of VIM-2 role in

E-selectin-dependent cell adhesion.

Contrary to previous speculation, the binding site of selectins was identified as a series of novel unbranched long-chain sialylated polylactosamine (PLA) internally polyfucosylated structures.

VIM-2 antigen did not bind to E-selectin. Neither $SLe^x$, bivalent $SLe^x$, sialosyl dimeric $Le^x$ nor sialosyl trimeric $Le^x$ were present in neutrophils or HL60 cells. Therefore, none of those structures are physiologic ligands of E-selectin in lymphocytes.

## SUMMARY OF THE INVENTION

The instant invention relates to a class of isolated novel unbranched, long chain, 2→3 sialylated, internally α1→3 fucosylated polylactosamines. The penultimate N-acetyl glucosamine may be fucosylated.

The instant invention also relates to use of such isolated unbranched, long chain, sialylated, internally fucosylated polylactosamines, or derivatives thereof, to intervene in selectin-mediated phenomena. For example, suitable derivatives are those which are stable to rapid inactivation in vivo.

Moreover, the instant invention relates to methods for making such sialylated polylactosamines and derivatives thereof.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B present HPTLC profiles of the HL60 cell monosialoganglioside fraction separated by HPLC on an Iatrobead™ column.

Figure 1A: The monosialoganglioside fraction was prepared from 300 mL of packed HL60 cells as

described herein. The fraction was mixed with 500 µL of isopropanol: hexane: water (IHW), 55:40:5, v/v/v, sonicated and injected onto an Iatrobead™ column (6RS-8010, 0.4 x 30 cm) pre-equilibrated with IHW, 55:40:5. Gradient elution from that solvent to IHW, 55:25:20, was performed over 400 min at a flow rate of 0.5 mL/min. Two mL fractions were collected and a 5 µL sample from each fraction was spotted on high performance thin layer chromatography (HPTLC) silica gel plates (EM Science, Gibbstown, NJ). HPTLC was developed with chloroform/methanol/0.5% $CaCl_2$ (50:55:19), and bands were revealed by reaction with an orcinol-sulfuric acid reagent. A, B, and C denote TLC migration positions of (respectively) three types of $SLe^x$ GSL: NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ→4Glcβ→1Cer, NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4GlcNAcβ1→3 Galβ1→4Glcβ1→1Cer and NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4[Fucα1→3] GlcNAcβ1→3Galβ1→4Glcβ1→1Cer.

Figure 1B: The polar monosialoganglioside fraction of HL60 cells was separated on HPLC in the IHW solvent system as described herein. Bands were revealed by TLC blotting with E-selectin-expressing CHO cells metabolically labeled with $^{32}P$ (14). Lanes 1-16 correspond respectively to fractions 9,19,21,27,31,33,37,39,41,43,44,45,46,47,48 and 49 of Figure 1A. The right-hand lane is $SLe^x$ ceramide hexasaccharide. All E-selectin binding fractions were slow-migrating glycosphingolipids (GSL's) containing long-chain PLA. No band was eluted corresponding to an $SLe^x$-containing GSL (see Figure 1A), although those species are found abundantly in eluates from human carcinoma tissues (15). Major reactivity with E-selectin was observed in very polar fractions, beginning with fraction 43 (lane 10).

Figure 2 presents a comparison of E-selectin-binding monosialoganglioside fractions extracted from human neutrophils and HL60 cells.

About 100 mL of human neutrophils were extracted and the monosialoganglioside fraction thereof was prepared as described herein. The fraction was compared with a corresponding fraction prepared from HL60 cells by HPTLC followed by blotting with $^{32}$P-labeled E-selectin-expressing CHO cells (14). Lane 1, SLe$^x$ ceramide hexasaccharide. Lane 2, total Folch's upper-layer GSL's from HL60 cells. Lane 3, purified monosialoganglioside fraction from lane 2. Lane 4, purified monosialoganglioside fraction from Folch's upper-layer GSL's from human neutrophils. The quantity of ganglioside mixture used for lanes 3 and 4 was based on approximately equal numbers of HL60 cells and neutrophils. Lanes 5 and 6, same as lanes 3 and 4 but diluted 2x.

Figures 3A-3F depict reactivity of polylactosamines, before and after sialidase treatment, with various mAb's.

For each figure, lane 1, fraction 12.2; lane 2, fraction 12.2 after sialidase treatment; lane 3, fraction 13.1; lane 4, fraction 13.1 after sialidase treatment; lane 5, dimeric Le$^x$ (III$^3$FucV$^3$FucnLc$_6$); and lane 6, nLc$_6$. Figure 3A: immunoblotting with anti-Gal$\beta$1→4GlcNAc$\beta$1→3Gal mAb 1B2. Figures 3B-3D: immunoblotting with anti-Le$^x$ mAbs, SH1, FH2 and anti-SSEA-1, respectively. Figure 3E: immunoblotting with mAb PL82G2. Figure 3F: glycolipid bands revealed by reaction with an orcinol-sulfuric acid reagent.

Sialidase treatment of fractions 12.2 and 13.1 was performed by incubation of 1 $\mu$g of glycolipid dissolved in 20 $\mu$L of 0.1 M sodium acetate (pH 4.5)

containing 0.02 units *Clostridium perfringens* sialidase at 37°C for 2 hr. Five $\mu$L of the reaction mixture was spotted onto a TLC plate and washed with water. The plate was dried and developed for 20 min in C:M:$CaCl_2$ (50:55:19).

Figures 4A and 4B depict $^1$H-NMR spectra of myeloglycans that bind (fraction 14; Figure 4B) or do not bind (fraction 13-0; Figure 4A) to E-selectin.

The two spectra are characterized by several common features: (i) $\alpha$-anomeric signal at 4.875 ppm, diagnostic for Fuc$\alpha$1→3 linked to type 2 chain GlcNAc$\beta$1→3 residue; (ii) a broadened and distorted quartet assignable to H-5 of the same Fuc$\alpha$1→3 substitution; (iii) a duplet at 1.015 ppm assignable to the Fuc$\alpha$1→3 methyl group (H-6); (iv) duplets at 2.576 ppm for H-$3_{eq}$ of terminal NeuAc$\alpha$2→3 (32); (v) a singlet at 1.889 ppm for the N-acetyl methyl group of NeuAc$\alpha$2→3; and (vi) a $\beta$-anomeric signal at 4.174 ppm assignable to Glc$\beta$1→1Cer.

In contrast, there are clear differences between spectra of E-selectin non-binding fraction 13-0 and binding fraction 14: (i) compared to fraction 13-0, fraction 14 has a much more intense (2-3 times higher) signal at 4.875 ppm; (ii) the GlcNAc-1 signals at 4.736 ppm (assigned as VII-1) and 4.748 ppm (assigned as IX-1) were prominent for fraction 14 but absent or unclear for fraction 13-0; (iii) fraction 14 showed greater upfield shifting of the GlcNAc-1 resonance and gave a more complex pattern, that is, the presence of resonances at 4.748, 4.736, and 4.700 ppm, which may be assignable to 3-substituted GlcNAc-1, the presence in fraction 13-0 of a duplet at 4.741 ppm likely is due to 3-substituted GlcNAc-1; and (iv) the quartet assignable to H-5 of the Fuc$\alpha$1→3

substituent shows a more complex pattern in the spectrum of fraction 14 than of 13-0, the spectrum assignable to Gal-1 was more complex and broadened in fraction 14 than in 13-0, suggesting complex interaction with the internal substituent.

Figure 5 depicts part of a myeloglycan including the repeating GlcNAc-Gal subunit. Below the backbone are various groups which can substitute for the sialyl residue at $R^1$ and various groups which can substitute for a fucosyl residue at $R^2$.

Figure 6 depicts a synthetic scheme for obtaining a starting material (4) in the chemical synthesis of myeloglycan. EtSH is mercaptoethanol. Ac is the acetyl group. MeOH is methanol. NaOMe is sodium methoxide. $Bu_2SnO$ is dibutyltin oxide.

Figure 7 depicts a scheme for the chemical synthesis of $Le^x$ derivatives containing $CF_3$-Fuc or 5-S-Fuc. $BF_3$-$Et_2O$ is boron trifluoride diethyl etherate.

Figure 8 depicts a scheme for the chemical synthesis of $Le^x$ derivatives containing 1-S-Fuc or C-Fuc. DMSO is dimethylsulfoxide. $NaBH_4$ is sodium borohydride. pyr is pyridine.

Figure 9 depicts a continuation of the scheme depicted in Figure 8 wherein the triflate is treated to yield the desired products.

Figure 10 depicts a scheme for attaching the various derivatives to a linear linking molecule or tether.

Figure 11 depicts a scheme for synthesizing

dimeric and trimeric Le^ derivatives. $(Ph_3P)_3RhCl$ is tris(triphenylphosphine)rhodium(I) chloride. DBU is 1,8-diazabicyclo[5.4.0]undec-7-ene. MeOTf is methyl trifluromethanesulfonate.

Figure 12 depicts a scheme for the synthesis of the core of myleglycan. $Ac_2O$ is acetic anhydride.

Figure 13 depicts a scheme for the synthesis of myeloglycan. $CrO_3$ is chromium(VI) oxide. $HBBr_2 \cdot SMe_2$ is dibromoborane-methyl sulfide complex. Pd/C is palladium on carbon. $SO_3 \cdot NMe_3$ is a complex of sulfur trioxide and trimethylamine.

Figure 14 depicts schemes for synthesizing a multivalent myeloglycan structure. $Boc_2O$ is di-tert-butyl dicarbonate. DCC is 1,3-dicyclohexylcarbodiimide. TFA is trifluoroacetic acid.

Figure 15 depicts alternative methods for obtaining polyvalent myeloglycan structures by incorporation into a liposome (top) or by polymerization (bottom). The symbols are as provided in earlier legends.

Figure 16 depicts a scheme for making a stable polylactosamine derivative containing a terminal KDN residue.

## DETAILED DESCRIPTION OF THE INVENTION

As used herein, "isolated" indicates some level of intervention wherein biologically active molecules in situ are removed from the naturally occurring situs. Generally, isolation involves a

level of purification.

"Derivative" is a molecule having the same biologic properties of myeloglycan but carrying chemical changes to enhance one or more properties of myeloglycan such as prolonged half-life, high binding affinity, tissue specficity and the like.

"Stabilized" indicates a derivative which has substantiallly the same biologic effect as the native, parent material but has a longer in vivo half-life as compared to that of the native, parent molecule.

Also, "cell" in meant to indicate a biologic entity that carries myeloglycan or selectin at the surface thereof. The cell may or may not contain a nucleus.

The myeloglycans of the instant invention comprise a discrete class of carbohydrate found in, for example, cells of the immune system. The myeloglycans mediate various stages of adhesion of lymphoid elements to various other cells, such as endothelium.

Neither HL60 cells nor human neutrophils expressed GSL's containing an $SLe^x$ terminal epitope as isolated previously and characterized from human colonic and other carcinoma tissues (15,16). Many E-selectin-binding components eluted on HPLC were slow-migrating, extremely polar GSL's, some of which were characterized as having unbranched long-chain PLA backbone structures with a minimum of 4 N-acetyllactosamine subunits. The existence of pairs of structures, one binding to E-selectin, the other not (e.g. fractions 12 vs. 13-1 and 13-0 vs. 14) indicates that E-selectin binding is based on terminally $\alpha 2 \rightarrow 3$ sialylated, internally multiply fucosylated structures. A sulfate group is not involved in the physiological process of neutrophil binding to E-selectin. Analysis of GSL fractions of

HL60 cells and neutrophils indicates that myeloglycan structures (but not $SLe^x$) are the physiologic E-selectin binding epitope.

Suitable cells for obtaining myeloglycans are those known to express ligands which bind selectin expressed on, for example, endothelial cells and platelets. Thus, cells of the immune system, which are known to bind to activated endothelium, for example, and specifically, which bind by virtue of reacting with selectin, are likely to contain myeloglycans and are suitable starting materials. Accordingly, lymphocytes, such as neutrophils, and various publicly available cell lines of immune cell origin can be used to isolate myeloglycan.

The cells are isolated using known techniques, such as centrifugation of whole blood, passing blood through an affinity matrix containing a reagent which can capture the cells of interest, for example, an antibody specific to a cell surface molecule on the target cell and the like.

Alternatively, cell lines are cultured using known methods and reagents. The cells are passed at appropriate intervals and collected by centrifugation.

The highly polar glycosphingolipids (GSL's) of the cells are extracted by exposing lysed cells, for example, following exposure to freezing temperatures, in a solvent, such as a mixture of an alcohol, an organic liquid and an aqueous liquid. A suitable solvent is one which can be used in a gradient elution chromatographic procedure. A suitable alcohol is isopropanol (I), a suitable organic liquid is hexane (H) and a suitable aqueous liquid is water (W). A suitable solvent is IHW in a ratio of 55:50:25, v/v/v.

The cells are extracted repeatedly with a suitable volume of solvent. The extraction can be

assisted using a mortar and pestle or an electric blender. The fluid phase is passed through a filter to remove the particulate matter, such as by filtering through diatomaceous earth.

The extracts are combined and evaporated to dryness.

The residue is dissolved in a volume of an aqueous solvent, such as water. The resulting solution was Folch partitioned with six volumes of chloroform (C): methanol (M), 2:1, v/v. The lower phase is repartitioned repeatedly with theoretical upper phase.

The upper phases are combined, the volume is reduced to a small volume, such as, about 10 ml, for example, by evaporation, and the sample is dialyzed against an aqueous buffer, such as distilled water, using dialysis tubing with a molecular weight cut-off of about 5000.

The dialysate is lyophilized and dissolved in a suitable liquid solvent in preparation for chromatographic separation, such as, chloroform (C):methanol (M):water (W), as described in (11). Thus, a suitable buffer is CMW at a ratio of 1:10:10, v/v/v. The solution is passed over a DEAE column, for example, having dextran as the inert carrier.

The monosialoganglioside fraction is eluted using the same solvent, for example, the 1:10:10 CMW solvent, but containing 0.03 M ammonium acetate.

The various monosialogangliosides can be separated on adsorption to, for example, a silica gel matrix. A suitable matrix is IATROBEADS™, and a suitable solvent is IHW, as taught in (12 and 13). Hence the starting solvent can have a component ratio of 55:40:5, v/v/v of IHW, and elution occurs over a period of about seven hours at a flow rate of about 0.5 ml per minute wherein the solvent gradient

varies to a final composition of, for example, 55:25:20 of IHW, to obtain separation, as known in the art. As noted in the drawings herein, essentially pure species of monosialogangliosides can be obtained.

The various species can be separated further by acetylation and preparative high performance thin layer chromatography as described in (12) and (13).

Determination of whether a monosialoganglioside binds selectin can be accomplished in any of a variety of art-recognized means. For example, (14) teaches a blotting-type method wherein the separated species are exposed to labelled cells known to express selectin, such as activated endothelial cells. Numerous other models for monitoring cell adhesion are known in the art. (64)

It was determined that the sialyl $Le^x$ ($SLe^x$) structure does not have a role as a selectin ligand in immune cells and HL60 cells. That conclusion was obtained on analysis of the various species of sugars isolated, as described herein, from HL60 cells (obtained from the ATCC) which are known to bind to activated endothelium via selectin.

GSL's corresponding to $IV^3NeuAcIII^3FucnLc_4Cer$ ($SLe^x$ ceramide hexasaccharide), $VI^3NeuAcV^3FucnLc_6Cer$ ($SLe^x$ ceramide octasaccharide) and $VI^3NeuAcV^3FucIII^3FucnLc_6Cer$ (sialosyl dimeric or trimeric $Le^x$ ceramide nonasaccharide), originally isolated and characterized from human tumor tissues (see Table II for structures), all are absent from the HPLC eluate of HL60 cells (Figures 1A and 1B). $SLe^a$ also is not found in HL60 or neutrophil extracts. Instead, the entire E-selectin binding activity is associated with a series of slow-migrating components (Figure 1B). E-selectin binding patterns of GSL's from HL60 cells and human neutrophils are identical (Figure 2).

No binding activity was detected for ACFH-18 antigen (12) (Table III), which has 12 sugar residues, a 10-sugar backbone, five N-acetyllactosamine subunits and the VIM-2 epitope as the terminal structure.

The shortest E-selectin-binding GSL from HL60 cells was purified and characterized as having the same backbone structure as ACFH-18 antigen, but with one more internal fucosyl residue. Thus, the E-selectin-binding GSL with the shortest carbohydrate chain was eluted at a position corresponding to ceramide-tridecasaccharide (13 sugar residues).

An analogous situation was found for fraction 13-0 and fraction 14. Both contain a backbone of 12 sugars with six N-acetyllactosamine subunits. Fraction 13-0 has the VIM-2 epitope as the terminal structure and does not bind to E-selectin. Fraction 14 has the same basic structure as 13-0, but contains one or two extra internally $\alpha 1 \rightarrow 3$ fucosylated residues and binds strongly to E-selectin.

The basis of structures 5-8 in Table III is as follows: (i) each of the structures, after treatment with sialidase, does not react with anti-$Le^x$ mAb SH1, but reacts strongly with anti-LacNAc mAb 1B2, since 1B2 does not react with $Le^x$, the results indicate that each of the structures contains a sialosyl-LacNAc terminus (NeuAc$\alpha$2→3Gal$\beta$1→4GlcNAc$\beta$1→3Gal$\beta$1→R) but does not contain an $SLe^x$ terminus (NeuAc$\alpha$2→3Gal$\beta$1→4[Fuc$\alpha$1→3]GlcNAc$\beta$1→3Gal$\beta$1→R); (ii) each of the structures, after desialylation, reacts strongly with mAb PL82G2, which defines the structure Gal$\beta$1→4[±Fuc$\alpha$1→3]GlcNAc$\beta$1→3Gal$\beta$1→4[Fuc$\alpha$1→3]GlcNAc$\beta$1→3Gal$\beta$1→4[±Fuc$\alpha$1→3]GlcNAc→; (iii) since the

E-selectin-binding components (fractions 13-1 and 14) showed much higher levels of Fucα1→3GlcNAc residue than did the non-binding components (fractions 12 and 13-0) on $^{1}$H-NMR (Figure 4), but do not contain an SLe$^{x}$ terminus, the minimal requirement for the binding structure is:

```
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAc;
 3                       3            3
 ↑                       ↑            ↑
α2                    Fucα1        Fucα1
NeuAc
```

(iv) the structures cross-react strongly with anti-SLe$^{x}$ mAb's, such as CSLEX, FH6, SNH3 and SNH4; and (v) sulfate groups were undetectable on Azure A staining on TLC.

Myeloglycans are found at the surface of neutrophils, other leukocytes and HL60 cells. Myeloglycans are found not only linked to ceramide, a sphingolipid, bound to cell membranes, but also can be linked to a carrier molecule, via, for example, a hydroxyl group. For example, the hydroxyl group may be that of serine or threonine residues of various cell membrane proteins or transmembrane proteins, such as those having a mucin-like domain, that is, having multiple repeats of a serine-rich or threonine-rich peptide. Multiple myeloglycan chains can be linked to such mucin-like core structures.

**TABLE I.** Functional group analysis of GSL fractions by mAbs and $^{1}$H-NMR.

| Fraction | mAb | | | | αFuc1→3-GlcNAc | Fuc-5 quartet | | | GlcNAc-1 doublet | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1B2 (LacNAc) | SH1 (Le$^{x}$) | PL82G2 | FH6 (SLe$^{x}$) | 4.875 ppm | 4.590 ppm | 4.594 ppm | 4.603 ppm | 4.748 ppm | 4.741 ppm | 4.73 ppm |
| 12-1 | – | – | – | + | | | | | | | |
| desialylated | ++ | – | + | – | | | | | | | |
| 13-1* | – | – | – | + | | | | | | | |
| desialylated | ++ | – | + | – | | | | | | | |
| 13-0 | – | – | – | + | + | + | | | | + | |
| desialylated | ++ | – | ++ | – | | | | | | | |
| 14* | – | – | – | ++ | +++ | | + | + | + | | + |
| desialylated | ++ | – | ++ | – | | | | | | | |

*Shows strong binding to E-selectin.

TABLE II. Glycoconjugates isolated and characterized from human tumors and containing SL epitope.

| No. | Structure | Presence in neutrophils and HL60 cells |
|---|---|---|
| 1 | Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 3<br>NeuAcα2 Fucα1 | – |
| 2 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 3<br>NeuAcα2 Fucα1 | – |
| 3 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 3 3<br>NeuAcα2 Fucα1 Fucα1 | – |
| 4 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 3 3 3<br>NeuAcα2 Fucα1 Fucα1 Fucα1 | – |

**TABLE III.** Major glycoconjugates present (on GSL's[2]) in HL60 cells and human neutropluls.

| No. | Structure | E-selectin binding |
|---|---|---|
| 5 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 ↑ NeuAcα2 (on Gal); 3 ↑ Fucα1 (on GlcNAc) | – |
| 6 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 ↑ NeuAcα2; 3 ↑ Fucα1; 3 ↑ Fucα1 | ++ |
| 7 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 ↑ NeuAcα2; 3 ↑ Fucα1 | – |
| 8 | Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer<br>3 ↑ NeuAcα2; 3 ↑ Fucα1; 3 ↑ Fucα1; 3 ↑ ±Fucα1 | ++ |

from that data derives the conclusion that the E-selectin ligand is at least a undecasaccharide bearing a terminal sialyl group and wherein at least two internal N-acetyl glucosamine (GlcNAc) residues are fucosylated. The most terminal GlcNAc residue, the penultimate GlcNAc of the backbone, is not fucosylated but as the backbone is lengthened, other internal GlcNAc residues can carry a fucosyl residue. While the size of the backbone is variable and may range to 40 residues or more, a suitable size to the backbone is from 8 to about 22 residues, wherein the backbone comprises multiple, polymerized N-acetyllactosamine subunits.

A suitable backbone size of a myeloglycan is one containing 4 to 6 N-acetyllactosamine units and with 2 or 3 $\alpha 1 \rightarrow 3$ fucosyl residues because of easier purification or synthesis, however, higher levels of binding to E-selectin may be obtained with myeloglycans with longer backbone chain lengths.

The ligand of P-selectin may vary somewhat from that of the E-selectin in terms of the number of N-acetyllactosamine units and fucosyl residues.

The sialyl and fucosyl residues, and the location thereof, provide the myeloglycan with the proper charge and configuration suitable for interacting with selectins.

The instant invention contemplates at least a second class of myeloglycans which carry the same characteristics of the class of myeloglycan described hereinabove except that the penultimate glucosamine is fucosylated. One or more other internal residues are fucosylated as well. The conditions for the length of the backbone as for the first class of molecules applies to the second class as well. Hence, the second class of myeloglycans has a minimal structure for binding to E-selectin the following backbone:

```
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAc
 3       3           3           3
 ↑       ↑           ↑           ↑
α2    Fucα1       Fucα1      ±Fucα1
NeuAc
```

Thus, the following structure binds to E-selectin:

```
Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCe
 3       3          3                3
 ↑       ↑          ↑                ↑
α2    Fucα1      Fucα1           ±Fucα1
NeuAc
```

The instant myeloglycans can be synthesized also using enzymes and suitable substrates or via a series of chemical steps.

The α1→3 fucosyltransferase (FT) from HL60 cells (myeloid type FT IV) (27,28) can create an α1→3Fuc linkage at the penultimate and internal GlcNAc's of PLA to form $Le^x$, dimeric $Le^x$ ($Le^x$-$Le^x$) or trimeric $Le^x$ ($Le^x$-$Le^x$-$Le^x$). However, the myeloid type FT IV cannot synthesize effectively $SLe^x$, i.e. create an α1→3Fuc linkage at the penultimate GlcNAc when the terminal α2→3 sialic acid is present. Under certain conditions, the myeloid type FT IV is capable of preferentially transferring an α1→3Fuc to an internal GlcNAc (29).

The α1→3FT capable of transferring Fuc to the penultimate GlcNAc when the terminal Gal is α2→3 sialylated has been distinguished from myeloid type FT IV and is identified as α1→3 FT VII (30,31).

The FT VII of neutrophils and HL60 cells may be active enough to synthesize internal fucosylated GlcNAc residues.

Thus, a myeloglycan can be synthesized using a 2→3 sialyl transferase and fucosyltransferases and a suitable backbone structure comprising at least eight sugar residues.

There are chemical synthetic schemes which can be used to synthesize native myeloglycan. The

schemes provided herein also are directed to making myeloglycan derivatives. Those schemes are applicable to synthesizing myeloglycan derivatives by substituting different reactants for those used to make the naturally occurring sugar.

A molecular linking group or tether can be attached to the reducing terminal of myeloglycan or derivatives thereof so that the molecules can be incorporated further to form multivalent structures, for example, by use of a starburst structure, liposomes or polymerization. A suitable tether or linking molecule is one which is bifunctional, carrying at one end a group reactive at least with GlcNAc of the myeloglycan backbone and at the opposite end of the linking molecule another generally reactive group. For example, a suitable linking molecule is a linear molecule carrying a reactive hydroxyl group at one end for reactivity with the GlcNAc residue and at the other end an amino group.

The chemical synthesis means for making myeloglycan also afford the opportunity to modify myeloglycan to obtain derivatives with desirable features, such as stability or enhanced reactivity. Derivatization of myeloglycans is constrained by the spatial relationship of the relevant substituents of native myeloglycan, that is, a terminal sialyl residue and multiple fucosyl residues.

Various modifications also can be made to the myeloglycan backbone. Moreover, changes to the backbone, as will be described hereinbelow, and the changes to the relevant substituents described hereinabove, can be combined in a single derivative molecule.

A pharmacophore search can be used to find alternative backbone or substituent structures, which may or may not comprise saccharide, which can

be used to configure or identify a molecule which binds selectin. First the myeloglycan pharmacophore is identified by structure-function studies, as described, for example, in the studies directed to SLe$^{x}$.(56) Distance parameters of the resulting functional groups are defined by use of NMR data, such as Nuclear Overhauser Effect (NOE), spectroscopy, methylation analysis and the like, coupled with conformational energy computations.

Based on the results of such physical studies, a minimum energy conformation model of myeloglycan can be obtained by computer assisted modeling, a number of software programs are known in the art. For example, a myeloglycan model was constructed based on HSEA (Hard Sphere Exo-Anomeric) calculations with the GESA (Geometry of Saccharides) program (Dr. Bernd Meyer, Department of Biochemistry, University of Georgia, Athens, GA) and visualized using the SYBYL molecular graphics program (Tripos Associates, St. Louis, MO) with computations performed on a Silicon Graphics IIRIS 4D/85 system (57).

The modeling demonstrated that the repeating N-acetyllactosamine core forms a helical structure with the carboxylate of sialic acid and the three vicinal hydroxyls of the internal fucose residues presented on that longitudinal structure in a specific spatial relationship. The conformation comprises the following glycosidic torsion angles ($\Phi/\Psi$): NeuAc$\alpha$2$\rightarrow$3Gal ($-170^\circ/-7^\circ$), Gal$\beta$1$\rightarrow$4GlcNAc ($54^\circ/9^\circ$), Fuc$\alpha$1$\rightarrow$3GlcNAc ($49^\circ/24^\circ$), GlcNAc$\beta$1$\rightarrow$3Gal ($57^\circ/-10^\circ$) and Gal$\beta$1$\rightarrow$4Glc ($55^\circ/2^\circ$).

The spatial dispositions of those functional groups are used to construct a model and a pharmacophore. Against that template, a synthetic molecule comprising a polymer or a single monomer can be substituted for the polylactosamine backbone

or molecule per se carrying the appropriate charges, hydrophobicity and the like of the relevant backbone elements and substituents in the same spatial organization as found in the native molecule to enable interaction of the substitute with selectin.

Alternatively, a search of available molecules approaching or having the necessary physical characteristics may reveal one, which although chemically unrelated, nevertheless may function as a substitute for the myeloglycan backbone or myeloglycan per se.

For example, the Fine Chemicals Directory data base (FCD 91.1) can be searched using the MACCS-3D software (Molecular Designs, Ltd., San Leandro, CA). Compounds are screened initially in the 2-D mode and matched compounds then are evaluated in the 3-D mode. Lead compounds then are subjected to biologic evaluation to select those with greatest impact on selectin binding. The lead compounds are modified, as described hereinabove, for example, to maximize selectin inhibition. That very approach was applied to $SLe^x$ and various non-carbohydrate inhibitors, such as a terpenoid compound, were obtained which successfully substitute for $SLe^x$ in biologic and functional assays.(56)

The derivatives are designed, for example, to enforce metabolic stability of myeloglycan without affecting the ability thereof to interact with selectins. Extensive structure-function studies on sialosyl $Le^x$ ($SLe^x$), which originally was thought to be a ligand for selectins, indicate that the structural elements required for $SLe^x$-selectin binding are the carboxylate group of sialic acid and the three vicinal hydroxyls of fucose (33). Therefore, an approach to construct derivatives is based, in part, on the replacement of a fucosyl residue by other functional groups, such as the more

stable $CF_3$ analogue of a fucosyl residue ($CF_3$-Fuc), a 5-thio-fucosyl residue (5-S-Fuc), a 1-thio-fucosyl residue (1-S-Fuc), a 6-trifluoromethyl fucose (61) or a carba-fucosyl residue (C-Fuc)(62) (Figure 5). In addition, the sialosyl residue can be substituted by an N-trifluoroacetyl or N-carbamyl group, or by simple anionic functional groups, including, for example, a carboxyl group, a sulfate group or a phosphate group, or by a modified sialic acid, such as deaminated neuraminic acid.

Both the fucose and sialosyl resides can be linked to the backbone via an S-glycoside bond rather than an O-glycosidic bond.

The sialic acid residue of various molecules can be a crucial element for activity of such molecules. Hence, removal of the sialic acid can lead to loss of activity. Sialidases (or neuroaminidases) are prevalent in body fluids and tissues and thus sialic acid-containing molecules can be unstable in vivo. It is believed that the S-$Le^x$ determinant has a half-life of about 10-15 minutes based on pulse-chase studies of labelled sialosyl oligonucleotides in mice.

A modified sialic acid residue as discussed hereinabove can enhance half-life if the sialic acid derivative is resistant to sialidases, particularly mammalian sialidases. An example is 2-keto-3-deoxy-D-glycero-D-galacto-nonulonic acid, also known as deaminated neuraminic acid or KDN. (63) The deaminated neuraminic acid can be obtained by a specific deamination of sialic acid or by using a deaminated neuraminic acid transferase with, for example, cytidine monophospho-deaminated neuraminic acid as a donor of the functional group for the enzyme. Any necessary fucosyl residues can be added to the backbone as described herein. A scheme for using KDN to obtain a stable polylactosamine is set

forth in Figure 16.

Since oligolactosamine constitutes the core structure of myeloglycan, a suitable starting material is the lactosamine derivative **4**, which can be prepared from a known disaccharide (34) **1** by sequential boron trifluoride etherate ($BF_3$-$Et_2O$)-induced thioglycosidation (35)(→**2**), deacetylation (→**3**) and stannylene-mediated regioselective allylation (36)(→**4**)(Figure 6).

Protected $Le^x$ trisaccharide derivatives can be prepared form starting material **4**. The 3-OH group of lactose and N-acetyllactosamine are known to be involved in intramolecular hydrogen bonding with 5'-O (37) which results in a decreased reactivity of that OH group(38). Thus, reaction of **4** with 3 molar equiv. of benzoyl chloride (BzCl) at low temperature (-45°C) yields the pentabenzoate **5**, whereas the conventional benzoylation affords the hexabenzoate **6** (Figure 7).

The requisite glycosyl donor **7** for derivative preparation is obtained from $CF_3$-Fuc(39) according to the procedure employed for fucose (40) which involves 1) formation of methyl α-glycoside, 2) benzylation, 3) acid hydrolysis and 4) trichloroimidation. The synthesis of other glycosyl donor **8** has been reported. (41)

Stereoselective α-glycosylation of **5** with **7** and **8** proceeds effectively in the presence of $BF_3$-$Et_2O$ to produce **9** and **10**, respectively. In the case of 5-thioglycosylation, it is reported that the 1,2-cis glycoside predominates even in the presence of the 2-O-acetyl group.(41)

1-S-Fuc and C-Fuc are introduced by substitution reactions of triflate using **14** (42) and **15** (43) as nucleophiles, respectively. Figure 8 summarizes the preparation of the triflate **13**, which involves the epimerization of the 3-OH group in **5** by

an oxidation (→**11**) and reduction (→**12**) sequence.

After generation of a thiolate (from **14**) and an alcoholate (from **15**) by treatment with sodium hydride, substitution reaction of **13** leads to the formation of **16** and **17**, respectively (Figure 9).

The aminohexyl linking molecule or tether is introduced to a reducing terminal of each $Le^x$ trisaccharide derivative **9, 10, 16** or **17** by glycosylation of **18** (44) using methyl trifluoromethanesulfonate (MeOTf)(45) as a promoter (Figure 10).

With the monomeric $Le^x$ derivatives (**9, 10, 16** and **17**) and those with a linking molecule or tether (**19-22**) readily available, the dimeric framework is assembled as shown in Figure 11. Selective removal of the allyl protecting group **19-22** through isomerization with $(Ph_3P)_3RhCl$ leads to the 3'-OH disaccharides which react with glycosyl donor **9/10/16/17** in the presence of MeOTf affording the corresponding dimeric $Le^x$ derivatives.

Reiteration of the deallylation and coupling procedures leads to the corresponding trimeric derivatives (Figure 11). Further deallylation of the trimers provides the proper acceptors for the next glycosylation.

Figure 12 provides the continuation of the buildup toward tetralactosamine core **B**. Thus, glycosylation of trimeric $Le^x$ derivative **A** of Figure 11 with **6** is followed by dephthaloylation using hydrazine hydrate, which concomitantly removes acyl protecting groups, and subsequent N,O-acetylation affords **B**. Selective removal of the allyl protecting group from **B** furnishes monohydroxyglycoside **C**.

The allyl functionality in **B** is transformed to a carboxyl group either by ozonolysis or by a hydroboration and oxidation sequence

(46)(Figure 13). On the other hand, sulfated and phosphorylated analogues can be prepared from **C**. Thus, exposure of **C** to the $SO_3 \cdot NMe_3$ complex in anhydrous pyridine (47) provides the sulfated derivatives, and phosphorylation of **C** by phosphitylation with dibenzyl N,N-diisopropylphosphoramidite and 1H-tetrazole, followed by oxidation with 3-chloroperoxybenzoic acid (m-CPBA)(48), affords the phospholylated derivatives.

Finally, deacetylation of the protected derivatives followed by hydrogenolysis yields the target myeloglycan derivatives.

The myeloglycan derivatives can be manipulated further through an amino functionality of the linking groups or tethers.

For example, oxidation of the trifunctional molecule **24** obtained from commercially available tris(3-hydroxypropyl)aminomethane (**23**) to the tris(carboxylic acid), followed by esterification with N-hydroxysuccinimide, yields the tris(active ester) **26** (Figure 14). A typical coupling reaction between **26** and myeloglycan derivatives provides the trivalent derivative **27**. The Boc group can be cleaved by acidolysis for further derivation to starburst structures.

To obtain liposomes, commercially available 2-tetradecylhexadecanoic acid (**30**) is converted into the active ester **31** and then coupled to myeloglycan derivatives (Figure 15, top). The resulting neoglycolipid **32** is used to prepare a liposome using known techniques.(55)

Free-radical polymerization of the acrylamide derivative **34**, prepared from **33** and myeloglycan derivatives, with acrylamide results in formation of the copolymer **35** in which composition and structure can be varied readily (Figure 15, bottom).

The instant methods for modifying sialyl residues and fucosyl residues to enhance the in vivo biologic activities of a molecule can be applied to any of the myeloglycans disclosed herein as well as to any molecule carrying a sialyl residue or a fucose residue. Thus, sialyl-Tn, sialyl $Le^x$, sialyl $Le^a$, $Le^x$, $Le^y$, $Le^b$, $GM^3$, $GD^2$, sialyl T and the like can be derivatized as taught herein.

Because the isolated myeloglycans are novel structures, the molecules can be used to generate antibodies thereto, which may be employed within the context of the instant invention to block the selectin-ligand binding reaction or for use as reagents for detecting myeloglycans. As to the various possible uses of myeloglycans, either a native myeloglycan or a derivative thereof may be used. As used herein, such antibodies include both monoclonal and polyclonal antibodies and may be intact molecules, a fragment of such a molecule or a functional equivalent thereof retaining binding specificity. The antibody may be engineered genetically. Examples of antibody fragments include $F(ab')_2$, Fab', Fab and Fv fragments.

Briefly, polyclonal antibodies are produced by immunizing an animal with the antigen of interest and subsequent collection of serum therefrom. Immunization is accomplished, for example, by a systemic administration, such as by subcutaneous, intrasplenic or intramuscular injection, into a rabbit, rat or mouse. It is preferred generally to follow the initial immunization with one or more booster immunizations prior to serum collection. Such methodology is well known and described in a number of references.

While polyclonal antibodies may be employed in the instant invention, monoclonal antibodies also are suitable. Monoclonal antibodies suitable for

use within the instant invention include those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (i.e., antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies are produced using methods known by those skilled in the art. Human antibodies and chimeric human-mouse antibodies are advantageous because of a theoretic reduced risk of generating xenogeneic antibodies thereto when administered clinically.

Monoclonal antibodies may be produced generally by the method of Köhler & Milstein (49 and 50), as well as by various techniques which modify the Köhler & Milstein method, see (51). Briefly, the lymph nodes and/or spleen of an animal immunized with one of the myeloglycans reactive with selectin are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin and, like the myeloma cells, has the potential for indefinite cell division. For immunization, it may be desirable to couple such myeloglycans to a carrier to increase immunogenicity. Suitable carriers include keyhole limpet hemocyanin, thyroglobulin, bovine serum albumin and derivatives thereof.

An alternative to the production of monoclonal antibodies via hydridomas is the creation of monoclonal antibodies expression libraries using bacteriophage and bacteria, see, for example, (52) and (53), or by in vitro immunization. Selection of antibodies exhibiting appropriate specificity may be performed in a variety of ways which will be evident to those skilled in the art.

A suitable antibody with specificity for a myeloglycan which binds selectin can be used as a reagent for detecting same in any of a variety of

art-recognized assay formats, such as RIA, ELISA and an assay monitored in a flow cytometer. Essentially a sample is exposed to the myeloglycan antibody. The myeloglycan antibody can be labelled. If labelled, following wash, presence of bound antibody is ascertained using an appropriate detector, such as scintillation counter or X-ray film for a radio-labelled antibody or a spectrophotometer for an enzyme-labelled antibody following exposure to a suitable substrate. If not labelled, a suitable second antibody is used, which second antibody may be labelled.

Obtention of purified sources of myeloglycans provides a method for inhibiting cell aggregation, immune cell aggregation, platelet aggregation and the like within a biologic preparation wherein aggregation is reliant on interaction of myeloglycan and selectin. The method comprises incubating a biologic preparation with at least one myeloglycan.

Purified or synthesized myeloglycan is precipitated, dialyzed to remove unwanted reagents and suspended in a physiologic buffer prior to use. The myeloglycan solution can be treated to provide a dry preparation, such as a powder, by lyophilization, for example.

Suitable biologic preparations include cell cultures and cell suspensions in biological fluids, such as blood, urine, lymph, synovial and cerebrospinal fluid. Myeloglycans generally will be incubated at a final concentration of about 0.1 to 1 M, and typically at about 0.2 to 0.5 M. Incubation is performed typically for 5 to 15 minutes at 37°C.

The instant invention also provides a method for inhibiting unwanted cell aggregation in a warm-blooded animal, such as a human. The method comprises administering to a warm-blooded animal an effective amount of at least one myeloglycan, the

myeloglycan inhibiting the binding of cells to sites expressing selectin. The instant myeloglycas can function as an anti-inflammatory agent.

The myeloglycans generally will be administered at a concentration of about 0.1 to 1 M and typically at about 0.2 to 0.5 M. It will be evident to those skilled in the art how to determine the optimal effective dose for a particular substance, e.g., based on in vitro and in vivo studies in non-human animals. A variety of routes of administration may be used. Typically, administration will be intravenous, intramuscular or intracavitary, e.g., in the pleural or peritoneal cavities, in the bed of a site of inflammation.

A myeloglycan can be combined with any of a variety of known excipients, fillers and the like known in the pharmaceutic arts as non-critical ingredients of a drug formulation aimed at enhancing properties of the final product. Any of a variety of standard pharmaceutic texts can be consulted, such as Remington's.

The myeloglycans also can be delivered by alterative means, such as by infusion pump, implant, patch, topically, by depot and the like. The myeloglycans can be contained within microspheres, such as microcapsules and liposomes. Standard methods for preparing same are known in the art (55).

Moreover, myeloglycan may be administered in combination with an immunotherapeutic or chemotherapeutic substance or in combination with an anti-inflammatory substance. When a combination of a myeloglycan and a substance is desired, each compound may be administered sequentially, simultaneously or combined and administered as a single composition. Dosages of each active ingredient are adjusted according to data obtained

in vitro, animal studies or empirical clinical studies, as is known in the art.

Diagnostic techniques, such as CAT scans, may be performed prior to and subsequent to administration to confirm the effectiveness of the inhibition of metastatic potential or inflammatory potential.

The instant invention now will be exemplified in the following non-limiting examples.

## EXAMPLES

### Example 1

HL60 cells were obtained originally from the American Type Culture Collection (ATCC) and grown in RPMI supplemented with 15% FCS. Cells were cultured continuously in roller bottles and harvested every four days. Altogether, 1100 mL of packed HL60 cells were divided into ≈300 mL packed aliquots. Normal (non-leukemic) human leukocytes (mostly neutrophils) were obtained from Japan Immunoresearch Laboratories, Takasaki City, Japan, wherein the cells were collected using an ex vivo circulatory system with a specific adhesion column. Frozen neutrophils were subjected directly to extraction of polar GSL's.

CHO cell tranfectants with E-selectin and P-selectin cDNA were established as follows. E-selectin cDNA in pCDM-8 was obtained from R&D Systems, Minneapolis MN. P-selectin cDNA was cloned from HEL cells (ATCC) based on the published sequence (2) and ligated in pRC/CMV (InVitrogen, San Diego CA). Chinese hamster ovary (CHO) DG44 cells (Dr. L.A. Chasin, Columbia University, NY) were cotransfected with E-selectin/pCDM-8 or P-selectin/pRC/CMV with pSV2/dhfr (ATCC) as

described previously (10). The transfected genes were amplified by stepwise selection for resistance to increasing concentrations of methotrexate (up to 3 μM and 5 μM for P-selectin and E-selectin expressors, respectively). P-selectin and E-selectin-expressing clones were isolated by cytofluorometry using anti-P-selectin mAb, such as, P1A, and anti-E-selectin mAb, such as, E12. The mAb's were established through immunization of BALB/c mice with NS-1 cells expressing P-selectin or E-selectin by standard procedures.

Example 2

Frozen cell pellets were extracted in five volumes of IHW (55:50:25 v/v/v) in a Waring blender for 5 min and suction filtered through Celite (Fisher Chemical Co.). The extraction was repeated three times.

Extracts were combined and evaporated to dryness under reduced pressure, the residue was dissolved in one volume water and Folch partitioned with six volumes of CM, 2:1. The lower phase was repartitioned three times with theoretical upper phase. Upper phases were combined, evaporated to a small volume (≈10 mL), dialyzed in distilled water through a Spectropore 5000 dialysis tubing and lyophilized.

The residue was dissolved in CMW 1:10:10 and applied to diethylaminoethyl Sephadex, as described previously (11). The neutral GSL fraction present in pass-through, monosialoganglioside fraction eluted with the same solvent containing 0.03 M ammonium acetate and disialoganglioside fraction eluted with the same solvent containing 0.13 M ammonium acetate were separated. Each fraction was concentrated, dialyzed and lyophilized.

The monosialoganglioside fraction was dissolved in IHW (55:40:5), introduced into an Iatrobead™ column and subjected to gradient elution with IHW, as described in the legend of Figure 1. A similar elution program was used previously for separation of monosialogangliosides (12,13). $IV^3NeuAcnLc_4Cer$, $VI^3NeuAcnLc_6Cer$, $IV^6NeuAcnLc_4Cer$, $VI^6NeuAcnLc_6Cer$, $IV^3NeuAcIII^3FucnLc_4Cer$ ($SLe^x$ ceramide hexasaccharide), $VI^3NeuAcV^3FucnLc_6Cer$ ($SLe^x$ ceramide octasaccharide) and $VI^3NeuAcV^3FucIII^3FucnLc_6Cer$ (sialosyl dimeric $Le^x$ ceramide nonasaccharide) were eluted at defined positions as shown by the arrows in Figure 1. Further purification of the E-selectin-binding GSL fraction was performed by acetylation and separation on preparative HPTLC as described previously (12,13). Separated fractions were deacetylated in CM-1% sodium methoxide in methanol, 2:1:0.1, for 10 min and desalted using known techniques.

Example 3

GSL fractions separated by HPLC as described herein were analyzed by HPTLC developed in various polar solvents (see legend of Figures 1 and 2). The TLC plate was blotted with metabolically $^{32}P$-labeled CHO cells expressing E-selectin or P-selectin as described previously (14) (see Figure 1 legend).

Example 4

To determine whether the GSL in question has the $SLe^x$ structure or $NeuAc\alpha2\rightarrow3Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3Gal\beta1\rightarrow$ structure, GSL's were desialylated by sialidase followed by TLC and then immunostaining with anti-$Le^x$ mAb's (e.g., SH1, FH2, anti-SSEA-1) or by immunoblotting with mAb 1B2 (which does not react with $Le^x$ but does react with

the LacNAc terminus Gal$\beta$1→4GlcNAc$\beta$1→3Gal$\beta$1→R). The procedure is described in the Figure 3 legend.

Example 5

The reactivity of each fraction was tested before and after sialidase treatment with mAb PL82G2 which binds to internally located Fuc$\alpha$1→3GlcNAc and various antibodies directed to $SLe^x$ such as FH6 (15), CSLEX (16), SNH3 and SNH4.

Example 6

Sulfate group was detected on TLC with the cationic dye, Azure A, as described previously (17,18). Sodium chlorate, which blocks biosynthesis of sulfate from PAPS was used to detect HL60 cell adhesion to E-selectin.

Example 7

$^1$H-NMR spectra were recorded with a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer and pulse programmer, operating in the Fourier transform mode with quadrature detection. Spectra were recorded at 328°K (for ACFH-18 antigen) or 325°K (for myeloglycan GSL fractions 13 and 14) (19) on deuterium-exchanged samples dissolved in 0.4 mL of dimethyl-sulfoxide-$d_6$ containing 2% $D_2O$ (20) and 1% tetramethylsilane as a chemical shift reference. Other parameters and data treatment were as described previously (19).

Example 8

As disclosed in (58)-(60), $SLe^x$ can affect cell aggregation in various animal models. In similar

fashion, myeloglycan can be shown to intervene in cell aggregation.

The highly metastatic BL6 clone of the B16 melanoma cell line (Dr. Jean Starkey, Montana State Univ., Bozeman, MT) was selected in syngeneic C57BL mice for high metastatic potential. C57BL mice were maintained in plastic cages under filtered air atmosphere and provided with water and food pellets. Cells were cultured in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal calf serum (FCS) and detached with phosphate buffered saline (PBS) containing 2 mM EDTA. Viability was tested by trypan blue exclusion test.

A suspension of BL6 cells (1-3 x $10^6$ cells/ml RPMI 1640 medium) was prepared and aliquots are incubated in the presence or absence of myeloglycans at various concentrations, at 37°C for 5-10 minutes. Following incubation, typically, 3 x $10^4$ or 2 x $10^4$ cells (with or without myeloglycan pretreatment) per 200 µl are injected via a tail vein into 8-week-old female mice. After 18-21 days, the mice are killed, the lungs are fixed in 10% formaldehyde in PBS (pH 7.4) and tumor cell colonies are counted under a dissecting microscope. Data on the number and the size of colonies are treated statistically by the analysis of variance (ANOVA) procedure. Colonies with a diameter of 1 mm or greater are considered large-size and those with a diameter less than 1 mm are considered small-size.

Colony number is reduced in animals receiving cells exposed to myeloglycan.

Example 9

Mice are exposed to radiolabelled myeloglycan by intravenous injection. Myeloglycan is radiolabelled using known synthesis methods such as

using a radiolabelled starting material as disclosed in the synthetic schemes described herein. For example, tritiated or $^{14}C$-labelled fucose or a fucose analog carrying $^{35}S$ can be used to label a myeloglycan. Varying amounts of labelled myeloglycan are administered to a host animal. Then any of a variety of known models of leukocyte adherence to endothelium can be used to provide a site for selectin expression, see Table 6.2 and references cited therein for a list of experimental models of vascular and tissue injury in (54).

Localization of labelled myeloglycan at the injury site can be assessed using known methods. Assessments can be taken at varying time points. Also, serum levels of myeloglycan can be ascertained. Such data will yield a suitable dose regimen to assure localization of adequate myeloglycan at the injury site.

Unlabelled myeloglycan at the thus empirically determined dose is administered to experimental hosts. The injury to obtain selectin expression is induced and then metabolically labelled leukocytes or tumor cells are administered to the treated host. The cells are labelled, for example, by culture in the presence of a radiolabelled nutrient, such as $^{35}S$ methionine. The degree of labelled cell binding to the injury site is assessed using known techniques.

Binding of leukocytes and transformed cells to the injury site is reduced in animals pre-treated with myeloglycan.

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All references cited herein are incorporated by reference in entirety.

An artisan will well recognize that various changes and modifications can be made to the teachings of the instant specification without departing from the spirit and scope of the instant invention.